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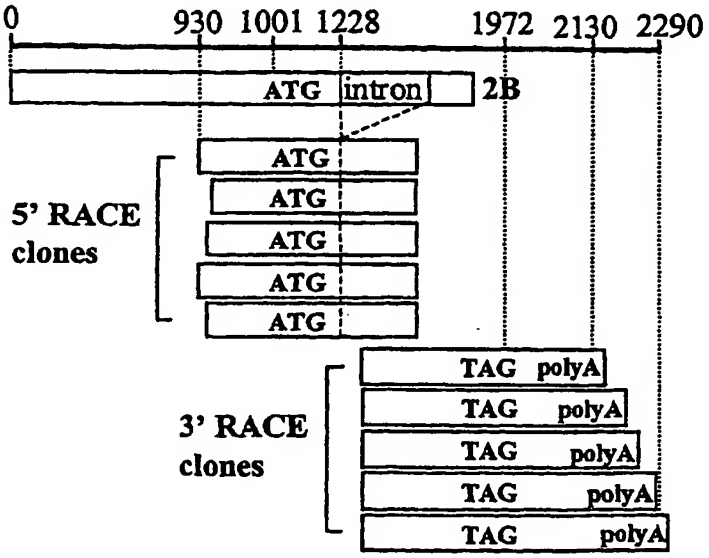
(54) Title: NEMATODE-UPREGULATED PEROXIDASE GENE AND PROMOTER FROM NEMATODE-RESISTANT MAIZE LINE Mp307



(57) Abstract: The present invention provides novel gene sequences which encode the peroxidase P7X gene and promoter isolated from maize inbred line Mp307. These novel gene sequences can be used in DNA constructs and transcription cassettes, which include the novel promoter of the present invention, a gene encoding a toxin, and a termination sequence. Additionally, these sequences can be used to produce transgenic plants resistant to nematode infection. Methods for nematode resistance in plants is also provided.

TITLE OF THE INVENTION

NEMATODE-UPREGULATED PEROXIDASE GENE AND PROMOTER FROM NEMATODE-RESISTANT MAIZE LINE Mp307

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a regular National application claiming priority from Provisional Application, U.S. Application Serial No. 60/167,229 filed November 24, 1999. The entirety of that provisional application is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to gene sequences that encode peroxidase genes. In particular, the present invention relates to the isolation and characterization of novel gene sequences which encode the peroxidase P7X gene and promoter isolated from maize inbred line Mp307. Additionally, this invention relates to methods of controlling plant-parasitic nematodes by application of recombinant DNA technology.

Background of the Technology

Nematodes are slender, worm-like organisms found in the soil almost anywhere in the world. A significant amount of the world's nematodes are plant parasitic, and are among the most devastating of the numerous pests that infest the

world's food crops. Generally, feeding nematodes have complex interactions with their host plants that last more than a month.

Each year, a majority of crop losses are caused by root-knot nematodes such as *Meloidogyne incognita*. Members of this genus have extensive host ranges and can parasitize monocots, dicots (eudicots), herbaceous and woody species, over 2000 different plant species in all (Hussey, 1985). Nematodes have been reported to cause crop loss equivalent to more than $6 billion in the United States and more then $100 billion around the world. (U.S. Patent No. 5,051,255).

With root-knot nematodes, infection of the plant occurs after juveniles (J2) hatch in the soil, invade the root, and migrate intercellularly to areas of differentiation and begin to set up a feeding site in xylem parenchyma cells in the vascular cylinder (Hussey and Williamson, 1998 and von Mende, 1997). This feeding site is established by injecting glandular secretions into the root cells via the nematode's stylet. (Bleve-Zacheo and Melillo, 1997). The nematode quickly becomes sedentary endoparasites, thereby losing its ability to reinfect. (von Mende, 1997). During this parasitism, plant cells become hypertrophic and multinucleate, which is the result of early nuclear division without cytokinesis. These multinucleate cells, called giant (nurse) cells are formed very early after infection. Eight nuclei can be found within 48 hours of nematode infection. (Wiggers, et al., 1990). The giant cells are metabolically active and serve as the nutritive source for the developing nematode. Qualitative and quantitative changes in giant cell gene expression has been hypothesized to accommodate the demands of the nematode (Bleve-Zacheo and Melillo, 1997). Thus, root-knot nematodes

cannot continue to develop normally without the induction and maintenance of these giant cells. (Hussey, 1989).

Various methods have been used to control plant parasitic nematodes. These methods include quarantine measures, manipulation of planting and harvesting dates, improved fertilization and irrigation programs that lessen plant stresses, crop rotation and fallowing, use of resistant and tolerant cultivars and rootstocks, organic soil amendments, and biological and chemical control. (Atkinson, 1992) Today, most of the plant-parasitic nematodes are controlled by chemical nematicides. These compounds are generally very toxic and have been suspected of causing environmental damage. For example, nematicides such as aldicarb, ethoprop, and carbofuran have been determined to be highly toxic to mammals, birds, and fish. Because of this, and the growing concern about the possibility of ground water contamination, several nematicides have had their use restricted or rescinded all together.

Thus, there exists a long felt need for safe and effective methods of protecting plants, particularly crop plants, from infection by plant-parasitic nematodes. Biological or "natural" methods rather than methods dependent on the application of chemicals are especially important from the standpoint of economics and environmental concerns.

SUMMARY OF THE INVENTION

It is an object of the invention to provide an effective and safe means to control plant-parasitic nematodes.

It is also an object of the invention to provide a novel gene sequence which encodes the peroxidase P7X gene isolated from maize inbred line Mp307.

It is yet another object of the invention to provide a novel gene sequence which encodes the upregulated peroxidase-promoter for the peroxidase P7X gene isolated from maize inbred line Mp307.

It is a further object of the invention to provide a method of nematode resistance in plants.

It is an object of the present invention to provide nucleic acid constructs and transcription cassettes which provide for regulated transcription in plant tissue in response to nematode infection.

It is a further object of the invention to provide transgenic plants containing the nucleic acid constructs according to the present invention.

DESCRIPTION OF THE FIGURES

Figure 1 is a schematic illustration of the assembly of the P7X peroxidase gene using ten clones obtained by RACE and one clone obtained by genomic walking.

Figure 2 is a photograph of a Northern Blot analysis for the expression of P7X peroxidase in roots.

Figure 3 is the cDNA sequence of P7X peroxidase (SEQ ID NO:1).

Figure 4 is the DNA sequence of P7X peroxidase promoter (SEQ ID NO:2).

BRIEF DESCRIPTION OF THE INVENTION

The amount of resistance a plant has against nematode infection relates to the plant's effects on nematode reproduction. For example, completely resistant plants do not allow any nematode reproduction, whereas susceptible plants allow nematodes to multiply freely. Recently, studies on host suitability of *M. incognita* have been conducted in corn. Of the 64 commercial *Zea mays* hybrids tested, all were found to be excellent hosts. (Windham, et al., 1987). Only a few of the open pollinated and inbred lines displayed resistances of varying degrees to *M. incognita*. One of the lines that displayed resistance was maize inbred line Mp307 (Aung, et al., 1990 and Williams, et al., 1988), although the mechanism of this resistance is unknown.

Diverse mechanisms have been attributed to plants that exhibit nematode resistance. The genetics of resistance has been traced to single dominant genes and also to recessive or polygenic traits in a variety of plant species. (Fassuliotis, 1987 and Trudgill, 1991). One example of genetic resistance is the tomato. Tomatoes contain a single dominant resistance gene, *Mi*, which has been recently isolated. (Vos et al., 1998). In addition to genetic resistance, various other mechanisms of resistance have been observed. Examples include hypersensitive responses, like in glyceollin I production (Huang, 1985), and secretion of repellents (cucurbitacins), toxins (α-terthienyl), or other compounds which affect hatching (Dropkin, 1989). Additionally, the ability of nematodes to penetrate roots (Canto-Saenz, 1985) and overall plant nutrition have been demonstrated as determinants of nematode resistance. (Huang, 1985). Because functional giant cells are essential for

development of root-knot nematodes, host "unsuitability" may be related to the inability to form these giant cells. (Id.). Other defense response chemicals, including peroxidases, have also been implicated in nematode resistance. (Baldridge, et al., 1998).

Peroxidase is a class of proteins whose primary function is to reduce hydrogen peroxide or molecular oxygen in the presence of an electron donor. (Kim, et al., 1999). Plant peroxidases are known to play a major role in lignin formation and wound healing, and are believed to be involved in auxin catabolism and defense to pathogen attack. (Lagrimini et al., 1987 and Klotz, et al., 1998). Most plants posses numerous peroxidase isoenzymes (isozymes) whose pattern of expression is tissue specific, developmentally regulated, and influenced by environmental factors. (Lagrimini, et al., 1987). These isozymes are usually classified in three groups, anionic (pI 3.5-4.0), moderately anionic (pI 4.5-6.5), and cationic (pI 8.1-11) based on their physical properties, and are believed to perform different functions during plant growth and development (Kim et al., 1999).

Although numerous peroxidase functions remain unknown, there is growing evidence that peroxidases are involved in plant defenses against pathogens and pests. It is believed that peroxidase action can create chemical and physical barriers against invading pathogens via cell wall lignification and protein cross-linking, generation of cytotoxic compounds, and/or oxidizing compounds that are important for pathogen metabolism. (Klotz, et al., 1998). Additionally, peroxidases appear to be intricately related to hypersensitive reactions (HR) in

resistant plants. (Hammond-Kosack and Jones, 1996 and Walton, 1997). However, their true physiological roles remain unclear. (Kim, et al., 1999).

A hypersensitive reaction is observed in a resistant plant in response to attempted infection by specific pathogens. The plant's resistance response can include localized plant cell death (HR) plus initiation of a signal transduction cascade which includes the expression of additional host defense genes such as phytoalexins and pathogenesis-related proteins. (Walton, 1997). HR-induced localized cell death is generally restricted to those plant cells immediately adjacent to the infection site, which can restrict and/or limit infection by obligate parasites. Early stages of a hypersensitive reaction are also associated with the generation of activated oxygen species. Activated oxygen species can generate various responses in the plant cells including host cell death, strengthening of cell walls via lignification and protein cross-linking, generation of secondary signals and injury to the invading pathogen. (*Id.*).

Evidence for peroxidases' roles in pathogen/pest resistance has been noted in various plant species. Increased peroxidase activity has been observed in a number of resistant interactions involving plant-pathogenic fungal and bacterial interactions. (Schweizer, et al., 1989; Graham, et al., 1991; and Chittoor, et al., 1997) and have been observed after elicitation with pathogenic organisms. An increased level of anionic peroxidase activity was observed in resistant tomatoes after nematode inoculation. (Zacheo, et al., 1993). Transgenic plants of *Nicotiana tabacum* (tobacco), *N. sylvestris* (ornamental tobacco), *Lycopersicon esculentum* (tomato), *Liquidambar styraciflua* (sweetgum) and *Zea mays* (maize) expressing

tobacco anionic peroxidase have exhibited increased resistance to insects. (Dowd, et al., 1999). Different species of insects were fed transgenic leaves from the above plants. Some insects displayed increased mortality and decreased body weight compared to those insects fed control leaves. (Id.). Researchers suggested that resistance was achieved due to a combination of three peroxidase-generated factors: enhanced tissue toughness, decreased nutritional quality, and enhanced toxicity. The enhanced toxicity was considered to be the main factor.

It is believed that a specific nematode-upregulated peroxidase promoter could be very valuable, and the regulation of a phytotoxic gene by such a promoter could destroy nematode feeding sites and confer resistance. To date, no nematode-upregulated promoters have been cloned from monocots and individual peroxidase genes associated with nematode expression have not been characterized.

In this regard, one aspect of the present invention is the isolation and characterization of the gene sequence which encodes the peroxidase P7X gene isolated from maize inbred line Mp307 as well as the gene sequence which encodes the upregulated peroxidase promoter for the peroxidase P7X gene isolated from maize inbred line Mp307.

The full P7X peroxidase gene sequence was assembled from ten independent clones obtained by rapid amplification of 5'- and 3'- cDNA ends (RACE) and one clone obtained by genomic walking. In particular, the 3'- RACE was performed with Jprim (3'-GTGCCCCAACGCGCTGTCCACCATCAA) and 5' RACE with P75w1 (5'-GCCCTAGCAGAACTGTCCACGAAGGCCCGCCGAGC) gene-specific

primers. A schematic illustration of the assembly of the P7X peroxidase gene using these clones is shown in Figure 1.

RACE allows for the selective amplification of the 5'- or 3'- end of cDNA that has an adapter of the subject invention attached at both ends of the cDNA. By using a combination of an internal target DNA primer that is complementary to a portion of the nucleotide sequence of the target DNA and a primer that is complementary to the primer binding portion of the adapter, only the 5'- or 3'- end of any individual RNA is exponentially amplified during the polymerase chain reaction (PCR) step. The non-target cDNAs that lack the internal primer binding sequence that is specific to the target cDNAs are not efficiently amplified.

The 5'- and 3'- RACE amplification products obtained from PCR are then fused to create a full length cDNA. The 5'- and 3'- RACE products are purified and then mixed together in the absence of primers. The mixture is subjected to several rounds of PCR cycling in the presence of a DNA polymerase. The overlapping regions of the RACE products anneal and are then extended to generate the full length cDNA.

Genomic walking is a technique whereby overlapping DNA fragments are sequentially isolated in order to "walk" up or down a larger polynucleotide segment, such as a chromosome. In the first cycle of genomic walking, a subfragment from one end of the first clone is used to isolate clones that extend farther along the chromosome. (Lewin, 2000). In subsequent cycles, a new clone that has a restriction map that coincides at one end with the end of the previous

clone and has new material at the other end is selected. (*Id.*). Cycles of clone selection are repeated until the full length sequence is obtained.

Sequence analysis of the independent RACE clones obtained in the present invention revealed that P7X peroxidase mRNA could be polyadenylated at various sites. (See Figure 1). Further computer analysis revealed a putative signal peptide coding sequence. The P7X peroxidase was predicted to be almost neutral (pI = 6.74) with extracellular localization. As shown in Figure 3, the total P7X peroxidase gene sequence was estimated to be 1379 bp.

The P7X peroxidase promoter was isolated by genomic walking, (Padegimas, et al., 1998). An additional P75w2 (5'-AGATTCGGGCCCTGGCTCTGCTCCCCTGACGTGTC) nested primer was utilized to increase the walking specificity and identify any regulatory sequences that may be present. It can be seen in Figure 1 that the comparative sequence analysis of the clone (2B) obtained by genomic walking with the P7X peroxidase mRNA sequence revealed a 108 nt intron located at position 227 of the coding sequence. It was determined that this intron is bordered by unusual nonconsensus sequences, specifically 5'-GGGC---AGGG. The P7X peroxidase promoter was estimated to be 1000 bp in length, as shown in Figure 4.

In a further experiment, radioactively labeled probes were prepared using the 3'- noncoding region of the P7X peroxidase gene. Northern blot analysis of RNA isolated from nematode-inoculated and control maize Mp 307 roots that revealed the P7X peroxidase gene is up-regulated in nematode-inoculated roots. (See Figure 2).

It is believed that the P7X peroxidase gene and the P7X peroxidase promoter can be used to fight nematode infection in plants. Four independent scenarios are suggested for obtaining nematode resistance in plants.

In the first scenario, it is hypothesized that the P7X gene driven by the P7X promoter can be used to induce a hypersensitive resistance response in root-knot nematode-infected plant roots. The possible candidates to promote a plant defense response include peroxidase genes which are known to be involved in hypersensitive responses, including signal transduction-indueed resistance. Increased peroxidase activity has been observed in a number of resistant interactions involving plant-pathogenic fungal and bacterial interactions, such as the increased level of anionic peroxidase activity that has been discovered in resistant tomatoes after nematode inoculation. (Zacheo, et al., 1993). The P7X peroxidase gene of the present invention has been shown to be induced in the root-knot nematode-resistant maize inbred line Mp307. Therefore, it is believed that the P7X peroxidase gene, driven by it native P7X promoter or other nematode-inducible promoters, could be utilized to obtain nematode resistance in transgenic plants. Other possible gene candidates involved in plant responses to pathogen attacks are described in Stintz, et al. (1993).

In accordance with the second scenario, it is hypothesized that the P7X peroxidase gene can be fused to a chimeric promoter for constitutive expression, (e.g., using the 35S promoter from the cauliflower mosaic virus (CaMV) (Benfey and Chua, 1990) or for more regulated expression (e.g. tissue specific promoter, inducible promoter, etc.). The 3'- untranslated sequences could be from the P7X

gene or from another plant-recognized gene. It has been determined that the introduction of a constitutively expressed peroxidase gene in transgenics was correlated with enhanced insect resistance in those plants (Dowd et al., 1999).

In accordance with the third scenario, it is believed that the expresssion of a gene toxic to nematodes in feeding site cells could poison the infecting nematodes, thereby debilitating them, or, if they are still motile, induce evacuation of the plant's roots. The use of the P7X peroxidase promoter would ensure that the toxin gene is expressed only, or substantially only, in cells surrounding the nematodes. This specificity would avoid any potential deleterious effects to the plant. In this regard, nucleic acid constructs are proposed which would allow for the expression of a toxin in a plant in response to nematode infection and provide an active defense response to root-knot nematodes.

The term "operatively linked" as used herein refers to DNA sequences on a single DNA molecule which are associated such that the function of one is affected by the other. A promoter is operatively linked with a structural gene when it is capable of affecting the expression of that structural gene. In other words, the structural gene is under the transcriptional control of the promoter. The promoter is said to be upstream from the structural gene and the structural gene is said to be downstream from the promoter.

The term "constructs" or "transcription cassettes" includes, in the 5' to 3' direction, a structural gene or a series of structural genes operatively linked to the promoter sequence or element, and optionally a termination sequence including a stop signal for RNA polymerase. All of these regions should be capable of

operating in the cells of the tissue to be transformed. Any suitable termination sequence may be employed. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment.

A proposed nucleic acid construct of the present invention is formed of the P7X peroxidase promoter of the present invention operatively linked to a toxin specific for nematodes. The toxin is inserted downstream from and under the regulation of the P7X peroxidase promoter. The peroxidase promoter provides for the initiation of the transcription of the toxin when nematode infection occurs.

A wide variety of toxins could be used, and would be easily identifiable by one of ordinary skill in the art. Examples of such toxins for use in the present invention include genes encoding *Bacillus thuringiensis* toxins described in EP 517,367 A1, genes encoding proteinase inhibitors such as cowpea trypsin inhibitor described in WO 92/15690, and genes encoding proteins such as miraculin that effect nematode sensory behavior.

The term "transgenic" in relation to the present invention does not include a promoter in its natural environment in combination with its associated gene or series of genes in its natural environment. Thus, the term includes seedlings or plants incorporating a gene or a series of genes which may be natural or non-natural to the seedling or plant operatively linked to the inducible gene promoter sequence or element of the present invention.

A transgenic plant is one which has been genetically modified to contain and express foreign DNA sequences. As specifically exemplified herein, a transgenic plant is genetically modified to contain and express a DNA sequence

operably linked to and under the regulatory control of transcription control sequences which it is not normally regulated. Thus, when a DNA construct of the present invention is incorporated into plant cells, and these cells replicated to generate a plant, a transgenic plant is formed. As used herein, a transgenic plant also refers to those progeny of the initial transgenic plant which carry and are capable of expressing a toxin in response to nematode infection. Seeds containing transgenic embryos are encompassed within this definition.

Methods of making transgenic nematode-resistant plants involve providing a plant cell capable of regeneration. The plant cell is then transformed with a DNA construct, and a recombinant nematode-resistant plant is regenerated from the transformed plant cell on appropriate selective media depending on the plant and vector used for transformation. Any cell tissue capable of propagation may be transformed. Transformation can occur by a number of well-known methods suitable for forming a transgenic plant, such as by introducing a transcription cassette into a plasmid vector specialized for plant transformation via *Agrobacterium tumefaciens* or by biolistic microparticle bombardment. Other methods of transformation would be known by one of ordinary skill in the art. Methods of maize transformation and examples thereof are set forth in U.S. Patent No. 6,140,555 and U.S. Application No. 09/698,080, both of which are incorporated by reference in their entirety.

The P7X peroxidase promoter may be active in both monocots and dicots (eudicots), and techniques are well-known in the art for the induction of DNA into monocots as well as dicots (eudicots), as are the techniques for culturing such plant

tissues and regenerating those tissues. As a result, a wide variety of plant species can be used in accordance with the present invention.

In the fourth proposed scenario, it is hypothesized that the P7X peroxidase promoter can be fused to a gene encoding a toxin which is toxic to plants for the induced prevention of giant cell formation in root-knot nematode-infected plants. It is believed that the induction of a plant toxin gene could kill the plant cells that are involved in providing nutrients to the nematode or suppress the development of the giant cells which are critical for nematode survival and reproduction, thereby either killing the nematode or forcing it to leave the root.

The plant toxin gene can be chosen from variety of genes that are capable of killing plant cells, disabling parenchyma cell development into feeding cells, or promoting a plant defense response, such as a hypersensitive response, that leads to cell death and signal transduction-induced resistance. The toxic product may either kill the plant cell in which it is expressed or simply disable the cell so that it is less capable of supporting the pathogen. It is preferred, especially where the plant is a food plant, that the plant-toxic product be non-toxic to animals, and particularly be non-toxic to humans.

Specific examples of genes that are capable of killing or disabling a plant cell include genes encoding proteases (e.g. trypsin, pronase A, carboxypeptidase, variety of endoproteinases), genes encoding ribosome inactivating proteins (RIPs) (e.g. RIP encoding genes from *Phytolacca americana, Phytolacca insularis, Phytolacca dioica, Phytolacca dodecandra*), genes encoding nucleases (e.g barnase, RNase A, RNase T, RNase CL-3, variety of DNA exonucleases and

endonucleases including restriction nucleases), genes encoding lipases (e.g. lipases from porcine pancrease and *Candida cyclindracea*), genes encoding membrane channel proteins (e.g. glp F and connexins), genes encoding antibodies targeted to plant cell essential proteins (e.g. RNA polymerase, respiratory enzymes, protein kinases, Krebs cycle enzymes, cytochrome oxidase, aminocyclopropane-1-carboxylic acid synthase, and enzymes involved in the shikimic acid pathway such as enolpyruvyl shikimic acid-5-phosphate synthase), genes encoding antisense RNA ~~or ribozymes targeted to plant cell essential proteins~~ mentioned above, and genes encoding toxins from plant pathogens (e.g., phaseolotoxin, tabtoxin, and syringotoxin).

Examples of genes that can disable the development of plant cells into the giant cell feeding sites for root-knot nematodes include genes that encode antibodies targeted to nematode injected proteins that induce feeding site formation and genes that encode antibodies targeted to plant proteins, hormones, or signal elements (of signal transduction pathway) that are produced in response to nematode attack and which play a critical role in the giant cell feeding site formation.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

Example : Isolation of P7X Gene and Promoter

Plant Material

Zea mays (maize inbred line Mp307) seedlings were inoculated with 5000 root-knot nematode (race 4) eggs. Both infected and control plants were grown for 9 additional days under greenhouse conditions. RNA extracted from these plants was used for RNA Northern gel blot analysis, cDNA synthesis, reverse transcription (RT) PCR, and for generation of adaptor-ligated libraries (for RACE).

Plant Genomic DNA Isolation

Plant genomic DNA was isolated from *Zea mays* (maize inbred line Mp307) leaves. Polysaccharides were removed by ethanol precipitation.

RNA Isolation

Total RNAs were isolated from inoculated and control roots using TRIzol® reagent (Gibco BRL) and an additional polysaccharide precipitation with 30% ethanol. The mRNA was purified with Dynabeads® (DYNAL).

Northern Gel Blot Analysis

Ten μg of total RNA was separated by agarose gel electrophoresis containing glyoxal/dimethyl sulfoxide. The RNA was transferred to GeneScreen Plus (DuPont) membranes using a downward alkaline capillary transfer procedure and was fixed by a microwave oven. Hybridizations were performed in ExpressHyb (Clontech) solution supplemented with a [α-^{32}P]dATP-labeled probe. The probe was obtained using Stratagene Prime-It II random primer labeling kit.

Subcloning

PCR fragments were ligated into pBluescript II KS (-) vector followed by transformation in XL-1 *E.coli* competent cells, with growth and selection on LB medium containing ampicillin. Recombinant clones were selected by PCR. All of these techniques are amply exemplified in the literature and are well-known to those of ordinary skill in the art. Particular exemplification is found in Sambrook et al., 1989.

Sequencing and Analysis

Sequencing was performed using manual as well as automatic sequencing methods. Manual sequencing was performed with a Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham). Automatic sequencing was performed using a flourescent terminators kit (Perkin Elmer) and an ABI310 sequencing system. Sequence analysis was performed with Lasergene (DNASTAR Inc.), PSORT (Nakai, K., Genomenet, Kyoto, Japan) and SignalP programs.

RT PCR Analysis

cDNA was synthesized using mRNA isolated from inoculated and control roots. PERPROM primer was employed for first strand cDNA synthesis. The cDNA was amplified using PERPROM and one peroxidase-specific (Hprim, Jprim or Kprim) primer. The mRNAs from inoculated and control roots were used as templates for negative controls.

RACE

cDNA corresponding to inoculated and control roots were synthesized and amplified. 5'-GAAGAATTCTCGAGCGGCCGC$(T)_{19}$V; IDT® Inc. was used for initiation of first strand cDNA and an additional primer (5'-pGAAGAATTCTCGAGCGGCCGC; IDT® Inc.) was added for cDNA amplification. Jprim (5'-GTGCCCCAACGCGCTGTCCACCATCAA) was used for 3'- RACE and P75w1 (5'-GCCCTAGCAGAACTGTCCACGAAGGCCCGCCGAGC) was used for 5'-RACE.

Each adaptor-ligated cDNA library was prepared by the following method. The PCR reaction mix was extracted twice with phenol:chloroform (1:1; v/v). DNA was precipitated by the addition of 0.5 vol. of 6.0 M ammonium acetate, 20 μg glycogen (Boehringer Mannheim) and 2.5 vol. ethanol. The samples were then incubated for 20 min. at -80°C and centrifuged at 1600 xg in a microcentrifuge for 15 min. Each pellet was washed twice with 85% ethanol, air dried, and dissolved in 20 μl H_2O. Approximately 2.0 μg cDNA (amount was estimated fluorometrically) was polished with 12 U T4 DNA polymerase (New England BioLabs) in 40 μl T4 DNA polymerase buffer (1X) saturated with 250 μm dNTP for 30 min at 16°C. Polished cDNA was extracted with phenol:chloroform, precipitated, washed, and dissolved in water as described above. The cDNA was then ligated with 50 pmol of a walking adaptor for 8 hours at 16°C using 10 U T4 DNA ligase (Fermentas) in 20 μl reaction volume using ligation buffer containing 40mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10mM DTT, and 0.5 mM ATP. The

reaction was terminated by a 15 min. incubation at 75°C, then diluted to 39 μl. One μl Exo III (200 U, Fermentas) was added. The reaction mix was incubated for 12 hours at 37°C and then terminated by a 15 min. incubation at 75°C. Adaptor-ligated cDNA libraries were stored at 20°C.

RACE was performed in 50 μl total volume which contained 1X Tth PCR reaction buffer [40 mM Tris-HC1 (pH 9.3 at 25°C), 15 mM KOAc; Clontech], 200 μM dNTP (Boehringer Mannheim), 1.0 mM $Mg(OAc)_2$, 0.2 μM each adaptor-specific (APN) and gene-specific primers, 1.0 μl adaptor-ligated cDNA library and 1.0 μl 50X Advantage™ Tth polymerase mix. PCRs were conducted using thin-walled tubes in a thermocycler. The thermocycler was programmed for 36 cycles: 10 sec. at 95°C and 3 min. at 67-73°C (dependent on primer optimal annealing temp.) with a 4 min. extension for the last cycle.

Genomic Walking

Walking in genomic DNA was performed on genomic DNA isolated from maize inbred line Mp307 leaves. Polysaccharides were removed by ethanol precipitation. DNA concentration was estimated fluorometrically using Hoechst 33258 dye.

The walking adaptor consisted of two oligonucleotides (LN and Oligol) that are partially complementary to each other and form unique secondary structure; LN 5' CAGCAGAACGACGCCCCGCCGACAAGGACAGGT (longer adaptor strand), Oligol: 5' ACCTGTCCTGCGAAAGCAsAsAsA (shorter adaptor strand). Adaptor-specific primer APN was utilized in each RACE or walking experiment; APN 5' CAGCAGAACGACGCCCCGCCGACAA. The primer used

in genomic walking was P75w2 (5'-AGATTCGGGCCCTGGCTCTGCTCCCCTGACGTGTC).

Five libraries were prepared using five different restriction endonucleases: *Bam*HI, *Dra*I, *Eco*RV, *Sca*I, and *Ssp*I. For each library, 10 µg maize genomic DNA was digested in 200 µl reaction volume with 100 U of appropriate restriction enzyme for 4 h at 37°C buffer which also contained 0.1 mg/ml BSA and 0.3 mg/ml boiled RNase A. Following digestion with *Bam*HI, 8.0 µl 2.5 mM dNTP and 2.0 µl (10 U) Klenow fragment of *E.coli* DNA polymerase I were added and then further incubated for 15 min. at room temperature. Five microliters of each reaction mix was loaded onto a 1.0% agarose gel to determine digestion efficiency. The remaining DNA was extracted twice with phenol/chloroform (1:1; v/v) and then precipitated by addition of 0.5 vol. 6.0 M ammonium acetate, 20 µg glycogen (Boehringer-Manheim), and 2.5 vol. ethanol. After mixing, the tubes were incubated for 20 min. at -80°C and centrifuged at 16,000*g* in a microcentrifuge for 15 min. Each pellet was washed twice with 85% ethanol, air dried, and dissolved in 20 µl H_2O.

The shorter strand adaptor oligonucleotide (1600 pmol) was phosphorylated using 40 U polynucleotide kinase in 40 µl buffer saturated with 1 mM ATP for 1 h at 37°C and then terminated by 10 min. incubation at 75°C. The longer strand adaptor (LN; 1600 pmol) was added, and then the mixture was heated for 1 min. at 75°C and incubated for 1 h at room temperature for annealing. Adaptors were extracted twice with phenol/chloroform and then precipitated by addition of 0.1 vol. 3.0 M sodium acetate, pH 5.2, 20 µg glycogen, and 3 vol. ethanol following by

20 min. incubation at -80°C and 20 min. centrifugation in a microcentrifuge at 16,000g. The DNA pellet was washed twice with 85% ethanol, air dried, and dissolved in 20 µl H_2O.

One-half of the digested genomic DNA (approximately 5.0 µg) was ligated with 50 pmol adaptor for 20 h at 16°C using 10 U T4 DNA ligase in 20 µl reaction volume with a buffer. The reaction was terminated by 15 min. incubation at 75°C. It was then diluted with 180 µl H_2O. Exo III (1.0 µl; 200 U) was added and incubated for 20 h at 37°C. The reaction was terminated by 15 min. incubation at 75°C. Adaptor ligated genomic libraries were stored at -20°C.

Each PCR (50 µl total volume) contained 1X *Tth* PCR buffer [40 mM Tris-HCl (pH 9.3 at 25°C), 15 mM potassium acetate; Clontech], 200 µM dNTPs (Boehringer-Mannheim), 1.1 mM magnesium acetate, 0.2 µM each APN and 3Zeal primers, 1.0 µl (approximately 25 ng) library DNA, and 1.0 µl 50X Advantage *Tth* polymerase mix. All reactions were carried out in thin-walled tubes. PCRs were conducted in a thermal cycler programmed for 7 cycles at 2 sec. at 93°C and 3 min. at 72°C, followed by 32 cycles at 2 sec. at 93°C, 3 min. at 67°C, and 4 min. extension for the last cycle.

The invention of this application is described above both generically, and with regard to specific embodiments. A wide variety of alternatives known to those of ordinary skill in the art can be selected within the generic disclosure, and examples are not to be interpreted as limiting, unless specifically so indicated. The invention is not otherwise limited, except for the recitation of the claims set forth below. All references cited herein are incorporated in their entirety.

WHAT IS CLAIMED IS:

1. An isolated DNA sequence having the sequence of SEQ ID NO:1.

2. A vector comprising the isolated DNA sequence of Claim 1.

3. The isolated DNA sequence of Claim 1, wherein said sequence is capable of inducing a hypersensitive response in nematode-infected plant roots.

4. A host cell comprising the vector of Claim 2.

5. An isolated DNA sequence having the sequence of SEQ ID NO:2.

6. A vector comprising the isolated DNA sequence of Claim 5.

7. A host cell comprising the vector of Claim 6.

8. A DNA construct comprising, in the 5' to 3' direction of transcription, a promoter, a gene encoding a toxin positioned downstream from said promoter and operatively linked thereto, and optionally a termination sequence positioned downstream from said gene encoding a toxin and operatively linked thereto, wherein said promoter is a nematode-inducible promoter having the sequence of SEQ ID NO:2.

9. The DNA construct of Claim 8, wherein said promoter is activated by nematodes.

10. The DNA construct of Claim 8, wherein said toxin is toxic to nematodes.

11. The DNA construct of Claim 8, wherein said toxin is toxic to plant cells and is capable of killing or disabling a plant cell so that it cannot nutritionally support a nematode.

12. The DNA construct of Claim 8, wherein said gene encoding a toxin is selected from the group consisting of genes encoding trypsin, genes encoding pronase A, genes encoding carboxypeptidase, genes encoding ribosome inactivating proteins from *Phytolacca americana*, genes encoding ribosome inactivating proteins from *Phytolacca insularis*, genes encoding ribosome inactivating proteins from *Phytolacca dioica*, genes encoding ribosome inactivating proteins from *Phytolacca dodecandra*, genes encoding barnase, genes encoding RNase T, genes encoding RNase CL-3, genes encoding phaseolotoxin, genes encoding tabtoxin, genes encoding syringotoxin, and genes encoding *Bacillus thuringiensis* toxins.

13. The DNA construct of Claim 8, wherein said promoter is derived from maize inbred line Mp307.

14. A transcription cassette comprising, in the 5' to 3' direction of transcription, a promoter, a gene encoding a toxin positioned downstream from said promoter and operatively linked thereto, and optionally a termination sequence positioned downstream from said gene encoding a toxin and operatively linked thereto, wherein said promoter is a nematode-inducible promoter having the sequence of SEQ ID NO:2.

15. The transcription cassette of Claim 14, wherein said promoter is activated by nematodes.

16. The transcription cassette of Claim 14, wherein said toxin is toxic to nematodes.

17. The transcription cassette of Claim 14, wherein said toxin is toxic to plant cells and is capable of killing or disabling a plant cell so that it cannot nutritionally support a nematode.

18. The transcription cassette of Claim 14, wherein said gene encoding a toxin is selected from the group consisting of genes encoding trypsin, genes encoding pronase A, genes encoding carboxypeptidase, genes encoding ribosome inactivating proteins from *Phytolacca americana*, genes encoding ribosome inactivating proteins from *Phytolacca insularis*, genes encoding ribosome inactivating proteins from *Phytolacca dioica*, genes encoding ribosome inactivating proteins from *Phytolacca dodecandra*, genes encoding barnase, genes encoding RNase T, genes encoding RNase CL-3, genes encoding phaseolotoxin, genes encoding tabtoxin, genes encoding syringotoxin, and genes encoding *Bacillus thuringiensis* toxins.

19. A transgenic nematode-resistant plant comprising transformed plant cells containing the DNA construct of Claim 8.

20. A method of providing nematode resistance in plants comprising transforming plant cells containing a DNA construct comprising a transcription cassette, said DNA construct comprising, in the 5' to 3' direction of transcription, a promoter, a gene encoding a toxin positioned downstream from said promoter and operatively linked thereto, and optionally a termination sequence positioned downstream from said gene encoding a toxin and operatively linked thereto, wherein said promoter is a nematode-inducible promoter having the sequence of SEQ ID NO:2.

21. The method of Claim 20, wherein said promoter is activated by nematodes.

22. The method of Claim 20, wherein said toxin is toxic to nematodes.

23. The method of Claim 20, wherein said toxin is toxic to plant cells and is capable of killing or disabling a plant cell so that it cannot nutritionally support a nematode.

24. A DNA construct comprising, in the 5' to 3' direction of transcription, a promoter, a structural gene positioned downstream from said promoter and operatively linked thereto, and optionally a termination sequence positioned downstream from said structural gene and operatively linked thereto, wherein said promoter is not the natural promoter for said structural gene and said structural gene is a peroxidase gene having the sequence of SEQ ID NO:1.

25. The DNA construct of Claim 24, wherein said promoter is activated by nematodes.

26. A transcription cassette comprising, in the 5' to 3' direction of transcription, a promoter, a structural gene positioned downstream from said promoter and operatively linked thereto, and optionally a termination sequence positioned downstream from said structural gene and operatively linked thereto, wherein said promoter is not the natural promoter for said structural gene and said structural gene is a peroxidase gene having the sequence of SEQ ID NO:1.

27. The transcription cassette of Claim 26, wherein said promoter is activated by nematodes.

28. A method of providing nematode resistance in plants comprising transforming plant cells containing a DNA construct comprising, in the 5' to 3' direction of transcription, a promoter, a structural gene positioned downstream from said promoter and operatively linked thereto, and optionally a termination sequence positioned downstream from said structural gene and operatively linked thereto, wherein said promoter is not the natural promoter for said structural gene and said structural gene is a peroxidase gene having the sequence of SEQ ID NO:1.

29. The method of Claim 28, wherein said promoter is activated by nematodes.

30. A transgenic nematode-resistant plant comprising transformed plant cells containing the DNA construct of Claim 24.

31. An isolated DNA molecule comprising SEQ ID NO:1 operatively linked in proper reading frame to SEQ ID NO:2.

32. A vector comprising the isolated DNA molecule of Claim 31.

33. A DNA construct comprising, in the 5' to 3' direction of transcription, a promoter, a structural gene positioned downstream from said promoter and operatively linked thereto, and optionally a termination sequence positioned downstream from said structural gene and operatively linked thereto, wherein said promoter has the sequence of SEQ ID NO:2 and said structural gene is a peroxidase gene having the sequence of SEQ ID NO:1.

34. The DNA construct of Claim 33, wherein said promoter is activated by nematodes.

35. The DNA construct of Claim 33, wherein said promoter is derived from maize inbred line Mp307.

36. A transcription cassette comprising, in the 5' to 3' direction of transcription, a promoter, a structural gene positioned downstream from said promoter and operatively linked thereto, and optionally a termination sequence positioned downstream from said structural gene and operatively linked thereto, wherein said promoter has the sequence of SEQ ID NO:2 and said structural gene is a peroxidase gene having the sequence of SEQ ID NO:1.

37. A transgenic nematode-resistant plant comprising transformed plant cells containing the DNA construct of Claim 33.

38. A method of providing nematode resistance in plants comprising transforming plant cells containing a DNA construct comprising, in the 5' to 3' direction of transcription, a promoter, a structural gene positioned downstream from said promoter and operatively linked thereto, and optionally a termination sequence positioned downstream from said structural gene and operatively linked thereto, wherein said promoter has the sequence of SEQ ID NO:2 and said structural gene is a peroxidase gene having the sequence of SEQ ID NO:1.

39. The method of Claim 38, wherein said promoter is activated by nematodes.

FIGURE 1

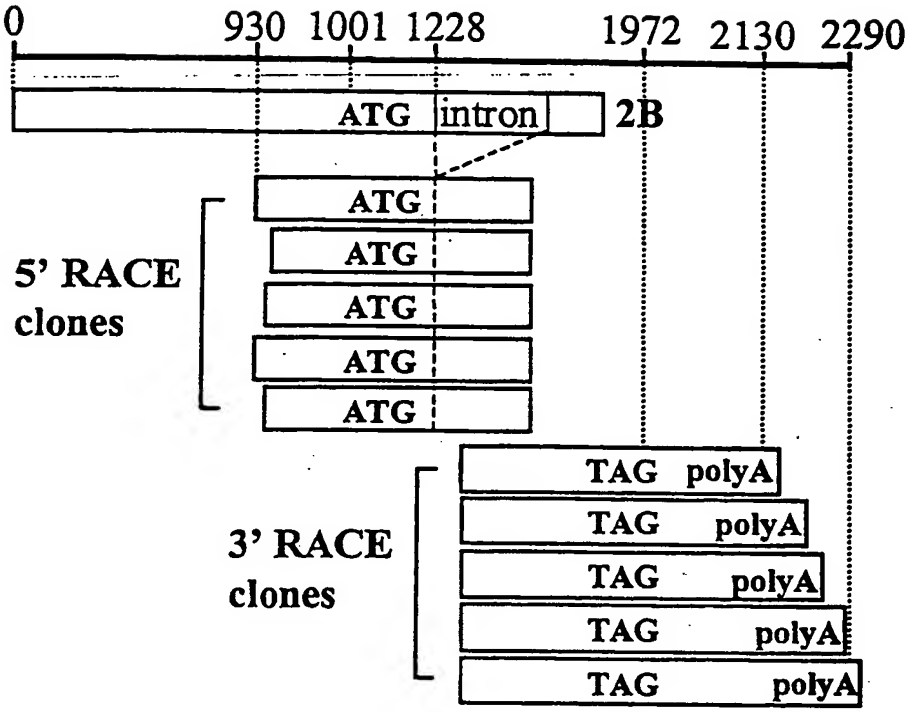


FIGURE 2

1 2

FIGURE 3

Sequence name - P7X peroxidase (cDNA sequence).

CACACACACCATATCACAAGCAAGCGCCAACGATCGAGCAGAAAGAAGATCGTCGAGATCGAGCATAAGCCATGGCGGCC
TCTGTTTCTGCCTCTTGCCTTATTAGCCTGTCGTCGTTGGCGGTGGTGCTGGTGGCGCTGGCGTCGGCGGCGTCGGCGCA
GCTGTCGTCGACGTTCTACGACAGGTCATGCCCCAACGCGCTGTCCACCATCAGGAGCGGCGTGAACTCCGCGGTGAGGC
AGGAGCCTCGCGTGGGGGCGTCGCTGCTCAGGCTCCATTTCCACGACTGCTTTGTCCGGGGCTGCGACGCGTCCCTTCTG
CTGAACGACACGTCAGGGGAGCAGAGCCAGGGCCCGAATCTAACTCTGAACCCAAGGGGCTTCGTTGTCGTGAACAGCAT
CAAGGCGCAGGTGGAGTCCGTGTGCCCGGGGATCGTCTCCTGCGCCGACATCCTCGCCGTGGCCGCCAGGGACGGAGTCG
TATCGCTCGGCGGGCCTTCGTGGACAGTTCTGCTAGGGCGAAGGGACTCTACCGCTTCATTCCCAGGACAGACAAGCGAC
CCCCCACCTCCGACGTCTAGCCTCCGACAGCTTTTGTCTGCGTATAACAAGAAGAATCTCAACCCAACCGACATGGTTGC
ACTCTCAGGAGCTCACACGATCGGACAGGCGCAGTGCTCGAGCTTCAACGACCACATCTACAACGACACCAACATCAACT
CCGCCTTCGCGGCGTCGCTCAGGGCCAACTGCCCCAGGGCAGGCAGCACCGCCCTTGCGCCGCTGGACACCACGACGCCC
AACGCGTTCGACAACGCCTACTACACCAACCTGCTGTCCCAGAAGGGGCTCCTGCACTCGGACCAGGAGCTCTTCAACAA
CGGCAGCACCGACAGCACGGTCAGGAGCTTCGCGTCCCAGCACGTCGGCCTTCAACAGCGCCTTCGCCACGGCCATGGTC
AAGATGGGCAACCTCAGCCCCCAGACCGGAACCCAGGGGCAGATCAGGCGCAGCTGCTGGAAGGTCAACTCGTAAACTAC
TAGCTACGTACTACGCCCAATGCAATGCGTTATGGCAGGCAGATCAGAGCCAATTAGTAATAAGGCCTCAGCTCGCTCTC
TACCTGTACGTGTGTGTGTGACTGGTGTTGGTCGAGTAAGTGTACGTACTACATGGATGGAGCAGAGAGAGAGAGAGATA
TATCGATTTGGCCACCTTATTATTGCATGCATATGTGTACTGTATTAGCACGACACTATTAGACACATTTATTAATGGTA
CCCATGCTATGCTTGTAAACGTACCCAGCCATTGACCTAGATATGACACTATAGGTCTAATCGTATTGCGGCTAACACCA
AAAAAAAAAAAAAAAAAAAA

Total length 1379 bp.

Locations/Qualifiers

1...1379
/P7X peroxidase cDNA sequence
72...1043
/sequence encoding P7X peroxidase

FIGURE 4

Sequence name - P7X peroxidase promoter.

ACTCACAACGCTAGCTTCTCTCTATAGTCTATAGAGTGCCGGTCATACCAATTATTTTATGGCACCCGTCGTGTGGCCCT
ATCATATATAAATACATATTTGCATATATTCCTTCTTAGCTACCATACACACAAAATTAGGCTCACGATATGGATGGATG
TTCAGCCATGCCGAGCCGAGCTGGCTTGTTAAAATAACGAACTGACTCGACAAAATCAGCTCAACTCAGTTTGATTGCAA
GTTTGAGCTGACTCGTTTAGCTCGTGAGACATAATAAAAAAATTATATATATAGTAATATATTCGATTACTAGATAGTTA
TAGACTAGTTTAAGACTAAAAAGGAGATATATAATACTCACAATTTCATATGTCGCCTCAATCGAACACCAGATCACAAT
TCATCACTTTTAGTTCATCCAACACAAGTACATGCTCCAGATCACAGTTCATCACTTTTTAGTTCATCCAACACAAGTAC
ATGCTTTGTTTTGCTGACAAATGCATGATTGCTTGTTCGAGCCAACAAGCTGGCTCATTAACAAACCGAGCCGAGATGCT
ACGTCAGCTCGTGATAAAATTCAAACGAACTGATCACATATAAGCCACGAGTATTTTGTCAGCCCTAGCTCATGATGTTA
TCCAGACACCTAACGTATATTGTATCACATGTGGGGCTAGTGTGCGTGTGGCTACCTGTGTACATGCACTGCATGACGAA
AATGGTTATTGCCGGCTTTCAGAGTCTAATTAAATAAATTAGCATGGACGTACTAATAATTCATAAGCTTGACGTCCGGT
TTGGATATATGTTATTTTCTGATCCATAGCTAGCTAGTAGTGACATGCATGCATGCATGATGAAGGCATCATAACATTAC
GGGCCCTAGCTAGCTAATAAATATGCATGTAGTGTATAGCATCGCCTTGCACACACACCATATCACAAGCAAGCGCCAAC
CATCGAGCAGAAAGAAGATCGTCGAGATCGAGCATAAGCCATGGCGGCCTCTGTTTCCGCCTCTTGCCTTATTGGCCTGT
CGTCGTTGGCGGTGGTGCTGGTGGCGCTGGCGTCGGCGGCGTCGGCGCAGCTGTCGTCGACGTTCTACGACAGGTCATGC
CCCAACGCGCTGTCCACCATCAGGAGCGGCGTGAACTCCGCGGTGAGGCAGGAGCCTCGCGTGGGGGCGTCGCTGCTCAG
GCTCCATTTCCACGACTGCTTTGTCCGGGCAAGTGAAGTCTCCATCATCTTGCATTCTGCTTTCTTTCTAGAAAACAAAC
ACCAGCAAAATTGAGGGCATTCGTTTAAAAAACTCTAACCCTAAATATTTCTGCAGGGCTGCGACGCGTCCCTTCTGCTG
AAC

Total length 1366 bp.

Locations/Qualifiers

1...1000
/P7X peroxidase promoter
1001...1228, 1337...1366
/partial P7X peroxidase coding sequence
1229...1336
/intron

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Published:
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: NEMATODE-UPREGULATED PEROXIDASE GENE AND PROMOTER FROM NEMATODE-RESISTANT MAIZE LINE Mp307

(57) Abstract: The present invention provides novel gene sequences which encode the peroxidase P7X gene and promoter isolated from maize inbred line Mp307. These novel gene sequences can be used in DNA constructs and transcription cassettes, which include the novel promoter of the present invention, a gene encoding a toxin, and a termination sequence. Additionally, these sequences can be used to produce transgenic plants resistant to nematode infection. Methods for nematode resistance in plants is also provided.

INTERNATIONAL SEARCH REPORT

Inter. ɔnal application No.
PCT/US00/30159

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 5/04; 15/09, 15/29, 15/82; A01H 5/00

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/278, 279, 287, 288, 298, 295; 536/23.1, 23.2, 23.6, 23.5, 23.3, 24.1; 435/69.1, 320.1, 468, 419, 199

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN CAS, WEST2.0

search terms: SEQ ID NO: 1-2, peroxidase gene or protein, nematode, resistance, peroxidase promoter

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,002, 068 A (PRIVALLE et al) 14 December 1999 (14.12.99), see entire document, especially columns 14-26, Examples 1-6.	1-4, and 24-38
Y	US 5,866,695, A (VIERLING et al) 02 February 1999 (02.02.99), see entire document, especially columns 13-17.	5-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 09 APRIL 2001	Date of mailing of the international search report 04 JUN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MEDINA A. IBRAHIM Telephone No. (703) 308 0196

Form PCT/ISA/210 (second sheet) (July 1998) ★

INTERNATIONAL SEARCH REPORT

Intern al application No.
PCT/US00/30159

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inter .onal application No.
PCT/US00/30159

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

800/278, 279, 287, 288, 298, 295; 536/23.1, 23.2, 23.6, 23.5, 23.6, 24.1; 435/69.1, 320.1, 468, 419, 199

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s)1-4 and 24-30, drawn to a DNA construct comprising SEQ ID NO:1, and a method of providing nematode resistance in plants by expressing said construct..
Group II, claim(s) 5-23, drawn to a DNA construct comprising the nematode-inducible promoter of SEQ ID NO:2 and a gene encoding toxin product, and a method for providing nematode resistance in plants by expressing said construct..
Group III, claim(s) 31-39, drawn to a DNA construct comprising SEQ ID NO:1 and 2, and a method of its use to induce nematode resistance in plants.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The invention of Group I, drawn to a first product requires the DNA sequence of SEQ ID NO:1 with any heterologous promoter which are not required by any of the groups.

The invention of Group II, drawn to a second product requires the nematode inducible promoter of SEQ ID NO:2 with a heterologous gene encoding a toxin which are not required by any of the other groups.

The invention of Group III, drawn to a third product requires SEQ ID NO:1 with SEQ ID NO:2 which are not required by any of the other groups.